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**13. ABSTRACT** ***(Maximum 200 Words)***

Targeting of adenovirus vectors, encoding for therapeutic genes, to tumor-specific receptors on breast cancer cells should result in specific killing of these cells. Targeting is necessary to prevent gene transfer in normal tissues resulting from the infection of normal cells by adenovirus. We have previously reported the use of an anti-knob antibody fragment (Fab), which prevents Ad infection, conjugated to folate to target adenovirus to folate receptor positive cells. In this report, the Fab has been conjugated on a large scale to an anti-EGFR antibody (425) and an anti-erbB-2 antibody (Herceptin) to yield Fab-425 and Fab-Herceptin conjugates, respectively. These conjugates were used to target adenovirus specifically to EGF and erbB-2 receptors on BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 breast cancer cells. In addition, the conjugates could specifically target adenovirus to the receptors in a hetergeneous cell population. *In vivo* studies were conducted which show that adenovirus can specifically target MDA-MB-468 cells using the Fab-425 conjugate. These results are significant in that they demonstrate that adenovirus vectors can be specifically delivered to breast cancer cells in a heterogeneous cell population and in vivo. This will be significant for treating disseminated breast cancer with adenovirus vectors.

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## Introduction

This research involves the targeting of adenoviral vectors to breast cancer for gene therapy applications through immunological methods. Adenoviral vectors have been widely used for cancer gene therapy applications because of their ability to accomplish efficient *in vivo* gene transfer. However, adenoviral vectors also infect a wide variety of normal tissues, mostly due to the widespread expression of the coxsackie-adenovirus receptor (CAR) which binds the adenovirus knob. Therefore, preventing the knob-CAR interaction while simultaneously introducing a novel ligand on the adenovirus for targeting breast cancer cells will result in an adenoviral vector which can accomplish breast cancer-specific gene transfer. I have previously described the use of the Fab fragment of an anti-knob antibody (1D6.14) conjugated to various ligands for targeting to cancer cells *in vitro* and *in vivo*. The purpose of this proposal is to conjugate breast cancer ligands to the Fab fragment and evaluate the resulting conjugates ability to target adenoviral vectors *in vitro* and *in vivo*. This final report will provide an overall summary of the work accomplished during the funding period with respect to the original statement of work.

## Body

### Approved Statement of Work

**Specific Aim #1: To conjugate the anti-knob Fab antibody to ligands that have a high affinity for cell surface receptors overexpressed in breast cancer cells.**

**Task 1:** Months 1-9: Conjugate EGF, FGF, and anti-erbB-2 antibody to the anti-knob Fab antibody and purify these conjugates. Validate these conjugates are made by SDS-PAGE analysis.

**Task 2:** Months 10-12: Validate the functionality of these conjugates using receptor inhibition assays and viral blocking assays.

**Specific Aim #2: To evaluate the efficiency and specificity of adenoviral-mediated gene transfer using these conjugates in vitro in breast cancer cell lines.**

**Task 1:** Months 13-24: Perform in vitro gene transfer studies using AdCMVLuc and the conjugates from Specific Aim #1 showing specific targeting to breast cancer cells that overexpress a particular receptor.

**Specific Aim #3: To evaluate the efficiency and specificity of adenoviral-mediated gene transfer using these conjugates in vivo in an animal model of breast cancer.**

**Task 1:** Months 25-36: In vivo gene transfer will be evaluated in tumor bearing nude mice using

AdCMVLuc and AdCMVLacZ. Each virus will be administered alone or after incubation with Fab or one of the conjugates to five mice. Gene expression will be evaluated either by a luciferase assay or X-gal staining.

*Conjugation of ligands to Fab.* Conjugation of EGF, FGF, and the anti-erbB-2 antibody (Herceptin) to the Fab was accomplished as proposed under **Specific Aim#1, Task 1**. In addition, an anti-EGFr antibody (425) was also conjugated to the Fab. All of these conjugates were confirmed by size-exclusion high performance liquid chromatography (HPLC). The HPLC analysis showed the formation of a high molecular weight species (~200 kDa for the Herceptin and 425 conjugates and ~60-80 kDa for the EGF and FGF conjugates) that were used in subsequent assays. This represented a small deviation from the Statement of Work which proposed to validate the conjugates by SDS-PAGE analysis. In addition, **Task 2** was not performed as described in the Statment of Work. It was decided that the functionality of the conjugates could be determined directly by moving to **Task 1 of Specific Aim#2**.

*Targeting of AdCMVLuc to breast cancer cells with Fab-EGF, Fab-FGF2, and Fab-Herceptin in vitro.* The studies described below directly address **Specific Aim#2, Task 1**. The breast carcinoma cell lines MDA-MB-134, MDA-MB-231, BT-474, MDA-MB-453, MDA-MB-468 and SK-BR-3 were obtained from the American Type Culture Collection (Rockville, MD). The BT-474 cells were maintained in RPMI 1640 with bovine insulin (10 μg/ml) and 10% fetal bovine serum (FBS); MDA-MB-134 cells were maintained in Leibovitz's L-15 medium with 20% FBS; MDA-MB-453 and MDA-MB-468 were maintained in Leibovitz's L-15 medium with 10% FBS; MDA-MB-231 cells were maintained in DMEM with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 1% MEM vitamins; and SK-BR-3 were maintained in McCoy's medium with 10% FBS. All media contained 1% L-glutamine. The AdCMVLuc, an E1-,E3-deleted Ad5 vector which expresses firefly luciferase driven by the CMV promoter, was generously provided by R.D. Gerard (University of Leuven, Leuven, Belgium).

To assess AdCMVLuc gene transfer or AdCMVLuc gene transfer targeted with Fab-FGF2, Fab-EGF, or Fab-Herceptin, cells were seeded 24 h prior to infection in 12 well plates with 4.8 x $10^4$ cells in triplicate. For Fab-EGF and Fab-Herceptin targeting experiments, cells were blocked with 300 μl/well of recombinant Ad5 knob (20 mg/ml), EGF (6.7 mg/ml) or Herceptin antibody (10 mg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugates. For Fab-FGF2 experiments, a neutralizing amount of Fab or a rabbit anti-FGF2 polyclonal antibody (Sigma Chemical Co., St. Louis, MO) was directly incubated with AdCMVLuc or

AdCMVLuc-Fab-FGF2 conjugate respectively for 30 min at room temperature for blocking gene transfer. For targeting experiments, AdCMVLuc ($4.5 \times 10^{10}$ plaque forming units (pfu)/ml) were preincubated with either PBS or the optimal amount (as determined by titering the conjugate against AdCMVLuc; data not shown) of Fab-FGF2, Fab-EGF, or Fab-Herceptin for 30 min at room temperature. The mixtures were diluted with OptiMEM (Gibco BRL, Grand Island, NY) to a final concentration of $4.8 \times 10^6$ pfu/ml and 200 µl added to each well (20 pfu/cell) and incubated for 1 h at 37°C. Cells were then washed with PBS, supplemented with complete media and incubated 24 h before lysis. Relative light units (RLU) were measured in a Berthold luminometer (Lumat LB 9501, Wallac, Gaithersburg, MD) using a luciferase assay kit (Promega, Madison, WI) according to the manufacturer's protocol.

**Figure 1** shows targeting of AdCMVLuc to BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells using the Fab-FGF2 conjugate. This figure shows that the luciferase expression in BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells infected with AdCMVLuc alone was $1.1 \times 10^6$, $2.5 \times 10^5$, $9.7 \times 10^4$, and $7.6 \times 10^5$ relative light units (RLU). Luciferase expression was inhibited >90% in all cell lines when the AdCMVLuc was neutralized with Fab. Infection with the Ad-Fab-FGF2 conjugate resulted in an increase in luciferase expression in all cell lines, which was shown to be specific by blocking with an anti-FGF2 polyclonal antibody. The ratio of targeted luciferase expression to non-targeted luciferase expression will be referred to as the targeting index (Ti). Thus, BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 cells have Ti values of 3.3, 3.7, 44.6, and 4.8 respectively. It can be noted that in general, if a cell line is resistant to Ad infection, it will have a higher Ti when targeting a heterologous receptor compared to a cell line that is easily transduced by Ad. Thus, the MDA-MB-134 cells have a Ti of 44.6 primarily due to the low level of luciferase expression when infected with AdCMVluc alone ($9.7 \times 10^4$ RLU).

Targeting of AdCMVLuc with the Fab-EGF conjugate to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, and SK-BR-3 cells is shown in **Figure 2**. This shows that infection of these cells with AdCMVLuc alone can be inhibited > 95% by blocking the adenoviral receptors on the cells with Ad5 knob. Also, targeting of AdCMVLuc with Fab-EGF to MDA-MB-468, MDA-MB-231, and SK-BR-3 cells resulted in Ti values of 11.5, 1.3, and 1.9, respectively, and specificity was demonstrated by inhibiting luciferase expression > 65% with an excess of EGF.

Targeting of AdCMVLuc with the Fab-Herceptin conjugate to BT-474, MDA-MB-231, MDA-MB-453, and SK-BR-3 cells is shown in **Figure 3**. This shows that infection of these cells with AdCMVLuc alone can be inhibited >95% by blocking the adenoviral receptors on the

cells with Ad5 knob. AdCMVLuc targeting with Fab-Herceptin was specific in MDA-MB-453 and SK-BR-3 cells as demonstrated by > 75% inhibition with an excess of Herceptin antibody. These cells had Ti values of 0.6 and 1.6 respectively. The BT-474 cells have a Ti value of 1.3; however, erbB-2 targeted gene transfer was not inhibited by an excess of erbB-2 antibody.

The previous results addressed and completed Specific Aim#2, Task 1, however, due to the complexities of working with the Fab-FGF2 and Fab-EGF conjugates, it was decided to end the investigation of the Fab-FGF2 conjugate at this point and switch from Fab-EGF to Fab-425 (an antibody conjugate that target EGFr). The complexities of the Fab-FGF2 and Fab-EGF conjugates included large scale production for in vivo studies and mitogenic activity of ligand binding to its receptor that could confound the targeting results. Thus, the next set of studies relate to **Specific Aim#2, Task 1** but were not explicitly in the Statement of Work.

*Specifically targeting AdCMVLuc to breast cancer cells in vitro using Fab-425 and Fab-Herceptin.* This is directly related to Specific Aim #2 of the application in order to confirm that the new conjugates made (Fab-425 and Fab-Herceptin) could specifically target AdCMVLuc to breast cancer cells *in vitro*. The statement of work said that these assays would be performed during the second year of funding. Although they were performed in the first year with the original conjugates, it was necessary to repeat these assays with the conjugates that will be used in vivo. Twenty-four hours prior to infection, the cells (MDA-MB-134, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, and SK-BR-3) were seeded in twenty-four well plates at a density of 1.2 x $10^4$ cells/well. The cells were then infected at 100 pfu/cell with AdCMVLuc that had been premixed with the optimal concentration of Fab-Herceptin or Fab-425 determined in the titrations (30 ng/1.2 x $10^6$ pfu). Infections were performed in the presence or absence of a saturating amount of Herceptin or 425 to inhibit adenoviral infection. Twenty-four hours after infection, the cells were harvested and assayed for luciferase expression (**Figure 4**). Figure 4 shows that the Fab-425 conjugate specifically targeted AdCMVLuc to the MDA-MB-468, MDA-MB-231, and SK-BR-3 cells. Targeting of AdCMVLuc to MDA-MB-468 with Fab-425 cells had the best targeting index (Ti; ratio of targeted luciferase expression to non-targeted luciferase expression) of ~5.8. The Fab-Herceptin conjugate targeted AdCMVLuc to BT-474, SK-BR-3 and MDA-MB-453 cells. The targeting index was ~1.1 for the BT-474 cells and ~1.8 for the SK-BR-3 cells. The MDA-MB-134 cells did not demonstrate specific targeting of AdCMVLuc with either conjugate. Thus, these conjugates were shown to specifically target AdCMVLuc to breast cancer cells that express EGFR, erbB-2 or both. The level of receptor expression on these cells was discussed in the first annual report.

*Targeting adenovirus in a heterogenous mixture of cells.* The ability of Fab-425 and Fab-

Herceptin to target an adenovirus encoding the green fluorescent protein (AdCMVGFP) was then evaluated in a mixed population of MDA-MB-468 (EGFr positive) and BT-474 (erbB-2) cells. The cells were seeded at 2 x $10^5$ cells/well in a six-well plate in a 20:80 ratio of MDA-MB-468:BT-474 to obtain ~45:55 ratio after 48 hours of growth. The cells were then infected twenty-four hours later at 10 pfu/cell with AdCMVGFP alone or AdCMVGFP that had been premixed with the optimal concentration of Fab-Herceptin or Fab-425 (30 ng/1.2 x $10^6$ pfu). In addition, cells were incubated with a total of 20 pfu/cell with AdCMVGFP mixed with both conjugates. After incubating for an hour, the conjugate/virus mixture was replaced with fresh media and harvested twenty four hours later for FACS analysis. EGFR expression and erbB2 expression was determined by incubating the cells with a mixture of 425 and Herceptin. The cells were then incubated with a mixture of goat anti-mouse and goat anti-human secondary antibodies that bind to 425 and Herceptin, respectively without cross-reactivity. The goat anti-mouse antibody was conjugated with allophycocyanin (APC), while the goat anti-human antibody was conjugated with phycoerythrin (PE). The cells were sorted by their APC and PE emissions by FACS and then analyzed for GFP expression. These results are summarized in **Figure 5**. The data are presented as the % positive gated cells and mean fluorescence intensity (MFI). In the EGFR positive, MDA-MB-468 cells, ~64% were positive for GFP with a MFI = 69 when infected with ADCMVGFP alone. This increased to ~81% when targeted with Fab-425 (MFI = 156) and decreased to ~21% (MFI = 51) when targeted with Fab-Herceptin. When a mixture of both conjugates were used, ~76% of the MDA-MB-468 cells were positive for GFP with a MFI = 100. This is in agreement with the luciferase results discussed earlier. The erbB-2 positive, BT-474 cells, were ~87% (MFI = 161) positive for GFP when infected with AdCMVGFP alone, this decreased to ~66% (MFI = 44) when infected targeted with Fab-425 and returned to ~88% (MFI = 114) when targeted with Fab-Herceptin. When a mixture of both conjugates were used, ~85% of the BT-474 cells were positive for GFP with a MFI = 77. Thus, the conjugates could still specifically target adenovirus to their appropriate receptor in the presence of another cell line not expressing that receptor.

*Targeting of AdCMVLuc to breast tumors in vivo.* This will describe studies that are directly relevant to addressing **Specific Aim#3, Task 1** from the Statement of Work. The Fab-Herceptin conjugate has not been evaluated in vivo thus far due to difficulties in forming established BT-474 (erbB-2 positive) tumors in mice. In addition, these studies do not evaluate targeting of AdCMVLacZ as described in the Statement of Work, but focus on in vivo delivery of AdCMVLuc. Athymic nude mice were injected with 5 x $10^6$ MDA-MB-468 cells subcutaneously followed by an i.v. injection of 1 x $10^9$ pfu of AdCMVLuc or AdCMVLuc premixed with the

optimal amount of Fab-425 three weeks later. Two days after adenoviral injection, the tumors and livers were removed from the animals and snap frozen in ethanol/dry ice. For luciferase analysis, the entire tissues were ground to a fine powder using a mortar and pestle. The powder was lysed in lysis buffer, subjected to three freeze thaw cycles, centrifuged, and luciferase expression determined using the Promega Luciferase Assay kit. Protein concentration for tissue were determined to standardize the luiferase expression per mg of protein. The results are presented as tumor-to-liver ratios of individual animals and are shown in **Figure 6**. The mean tumor-to-liver ratio for AdCMVLuc alone was 0.8 compared to 14.1 when the AdCMVLuc was targeted with Fab-425. In general, targeting AdCMVLuc with Fab-425 increased tumor luciferase expression about 3-fold when compared to non-targeted AdCMVLuc. Likewise, the Fab-425 "untargeted" the liver by decreasing the liver expression approximately 6-fold compared to the non-targeted AdCMVLuc. Thus, the Fab-425 could target AdCMVLuc to EGFr-expressing MDA-MB-468 tumors in vivo. These studies were directly related to completing the final task of the Statement of Work.

## Key Research Accomplishments

- Conjugation of EGF, FGF, 425, and Herceptin to the Fab anti-knob antibody.
- Evaluation of these conjugates for specifically targeting adenovirus (AdCMVLuc) to breast cancer cells in vitro.
- Large scale synthesis of immunological targeting reagents (Fab-425 and Fab-Herceptin) for *in vivo* targeting of adenovirus.
- Targeting of adenovirus to a heterogeneous population of breast cancer cells using Fab-425 and Fab-Herceptin.
- Demonstration that in vivo targeting of adenovirus can be accomplished using the Fab-425 conjugate to target AdCMVLuc to MDA-MB-468 tumors in nude mice.

## Reportable Outcomes

- Abstract presentation at the American Society for Gene Therapy 2nd Annual Meeting, Washington D.C., June 9-13, 1999.
- Oral presentation at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.

## Conclusions

In conclusion, the work presented in this report addressed four of the five tasks from the approved Statement of Work. The task that was not specifically addressed was answered by the results obtained from one of the other tasks. Specifically, the anti-knob Fab was conjugated to EGF, 425, FGF2, and Herceptin. These conjugates demonstrated specific targeting of AdCMVLuc to breast cancer cells in vitro. Targeting of adenovirus to EGFr and erbB-2 overexpressed on breast cancer cells with the Fab-425 and Fab-Herceptin, respectively, became the major focus of the project as the project evolved. It was demonstrated that Fab-425 could specifically target AdCMVLuc to EGFr positive MDA-MB-468 cells in vitro and that Fab-Herceptin could specifically target AdCMVLuc to erbB-2 positive BT-474 cells in vitro. Cell mixing experiments showed that AdCMVGFP could be delivered specifically to MDA-MB-468 or BT-474 cells in the presence of the other cell line using the appropriate conjugate. Finally, it was demonstrated that AdCMVLuc could be specifically targeted to MDA-MB-468 tumors growing in nude mice using the Fab-425 conjugate.

## Appendices

Figure Legend

Figures

Copies of Abstracts

Bibliography

## Figure Legend

**Figure 1.** Targeting of AdCMVLuc to the BT-474, MDA-MB-468, MDA-MB-134, and MDA-MB-231 breast cancer cells using the Fab-FGF2 conjugate. The cells ($4.8 \times 10^4$) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-FGF2 (52 ng per $9.6 \times 10^5$ pfu). A neutralizing amount of Fab or a rabbit anti-FGF2 polyclonal antibody was directly incubated with AdCMVLuc or AdCMVLuc-Fab-FGF2 conjugate respectively for 30 min at room temperature prior to infection for blocking gene transfer. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) (n = 3).

**Figure 2.** Targeting of AdCMVLuc to the BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, and SK-BR-3 breast cancer cells using the Fab-EGF conjugate. The cells ($4.8 \times 10^4$) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-EGF (13 ng per $9.6 \times 10^5$ pfu). Cells were blocked with 300 µl/well of recombinant Ad5 knob (20 mg/ml) or EGF (6.7 mg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate respectively. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) (n = 3).

**Figure 3.** Targeting of AdCMVLuc to the BT-474, MDA-MB-231, MDA-MB-453 and SK-BR-3 breast cancer cells using the Fab-Herceptin conjugate. The cells ($4.8 \times 10^4$) were infected at 20 pfu/cell with AdCMVLuc in the presence or absence of Fab-Herceptin (46 ng per $9.6 \times 10^5$ pfu). Cells were blocked with 300 ml/well of recombinant Ad5 knob (20 mg/ml) or Herceptin antibody (10 mg/ml) for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate respectively. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (RLU) (n = 3 for BT-474; n = 6 for other cell lines).

**Figure 4.** Targeting of AdCMVLuc to BT-474, MDA-MB-468, MDA-MB-134, MDA-MB-231, MDA-MB-453, and SK-BR-3 cells using the Fab-425 or Fab-Herceptin conjugate. The cells ($1.2 \times 10^4$) were infected at 100 pfu/cell with AdCMVLuc in the presence or absence of Fab-425 or Fab-Herceptin (30 ng per $1.2 \times 10^6$ pfu). Cells were blocked with an excess of 425 or Herceptin for 1 h at 4°C prior to addition of AdCMVLuc alone or the AdCMVLuc conjugate. Luciferase expression was assessed at 24 h post-infection and is shown as the mean ± s.d. of the relative light units (n=6).

**Figure 5.** Expression of GFP in a mixed population of MDA-MB-468 and BT-474 cells as analyzed by indirect immunofluorescence. The cells were seeded at a 20:80 ratio MDA-MB-468:BT-474 and infected with AdCMVGFP alone, AdCMVGFP premixed with Fab-425 or AdCMVGFP premixed with Fab-Herceptin. Twenty-four hours later the cells were harvested for analysis. The cells were first sorted to determine which expressed EGFR (EGFR+) or erbB-2 (erbB-2+). These populations were then sorted to determine the level of GFP expression (GFP in EGFR+ and GFP in erbB2+, respectively). The data are expressed as the percentage of positively gated cells (A) or mean fluorescence intensity (B).

**Figure 6.** Tumor-to-liver ratios in mice injected in the tail vein with either AdCMVLuc alone or AdCMVLuc targeted with the Fab-425 conjugate. Luciferase expression is determined as the relative light units per mg of tumor or liver for each individual mouse. The symbols represent the tumor-to-liver ratio for each mouse. Note that in mice injected with AdCMVLuc alone, three of the symbols are overlapping.

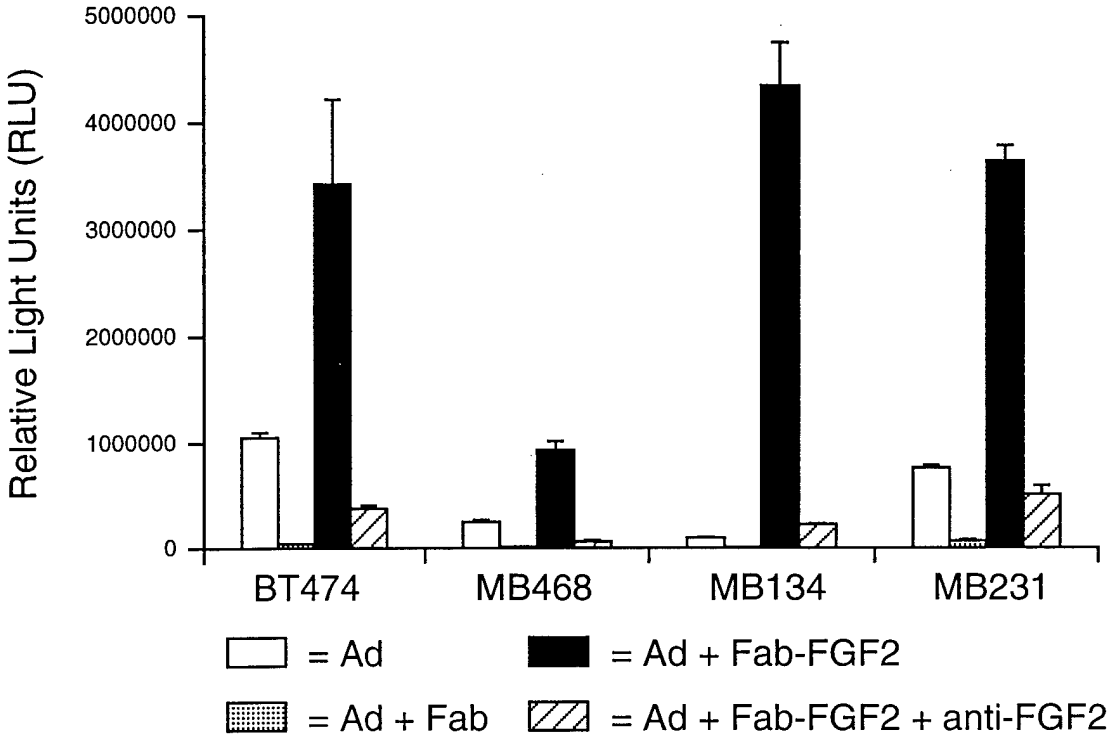
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Figure 1

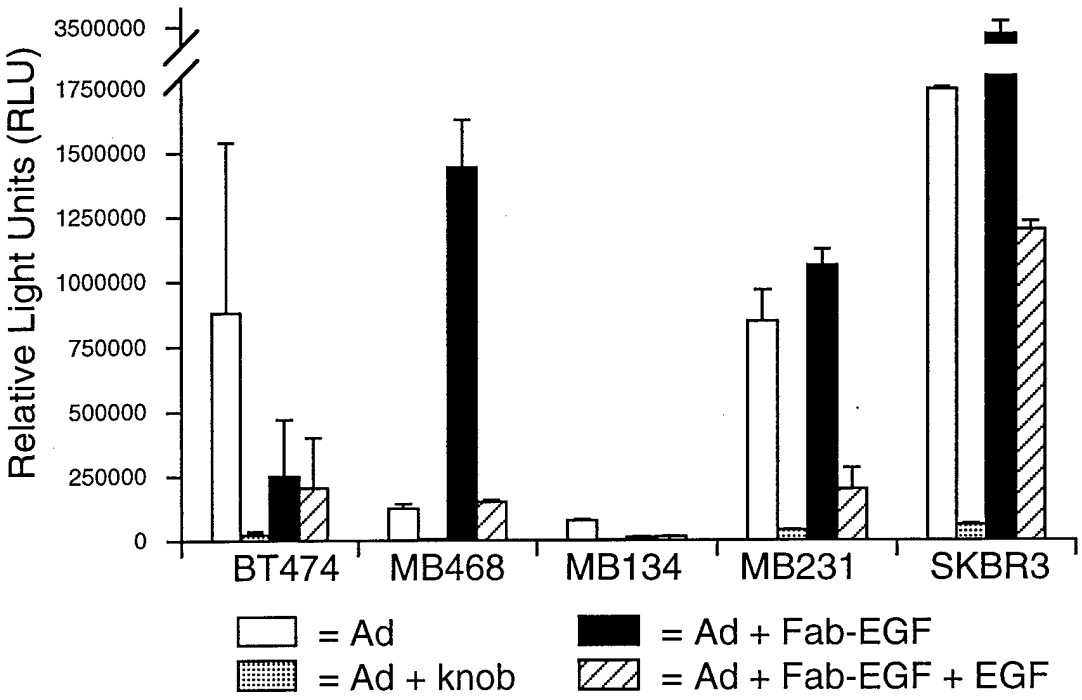
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Figure 2

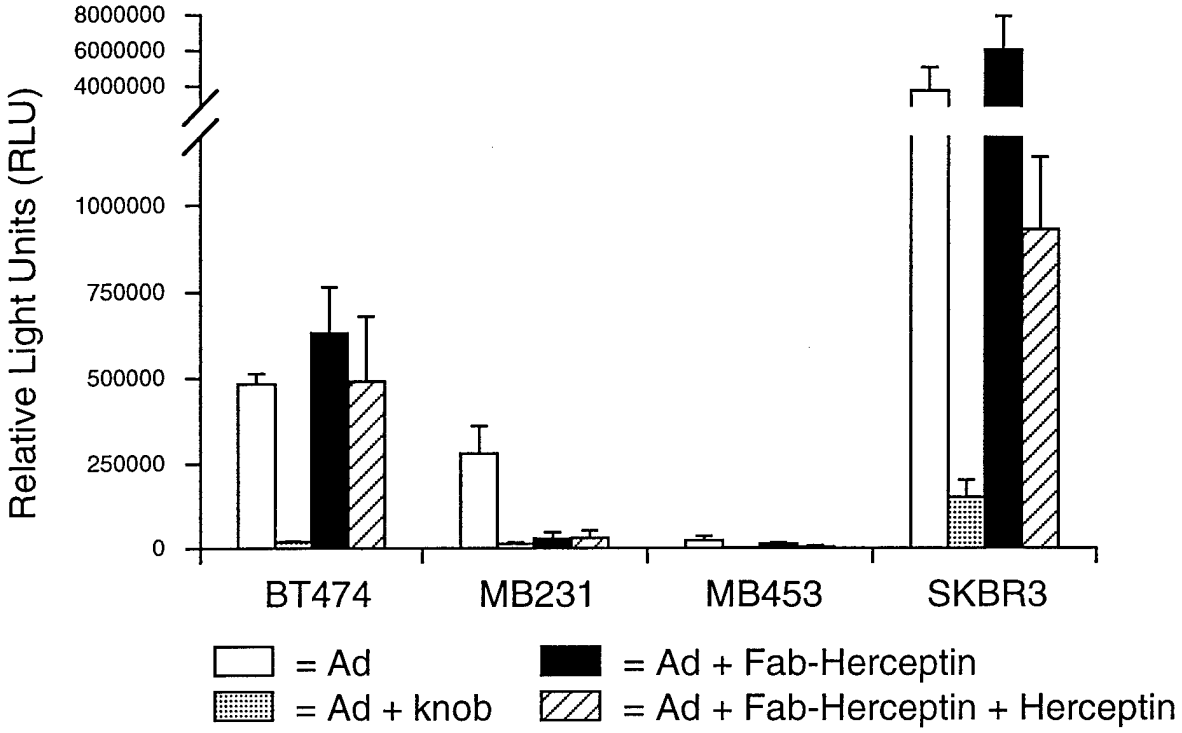
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Figure 3

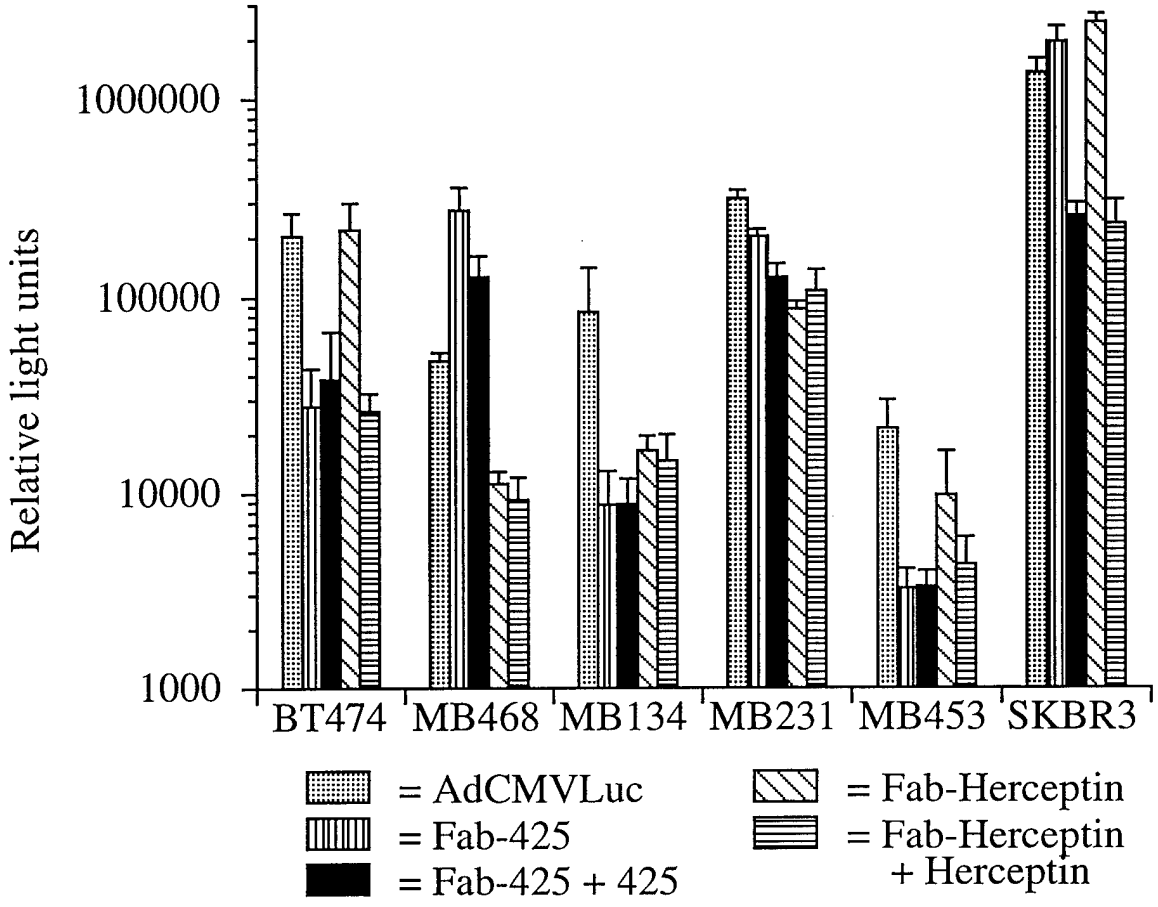
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Figure 4

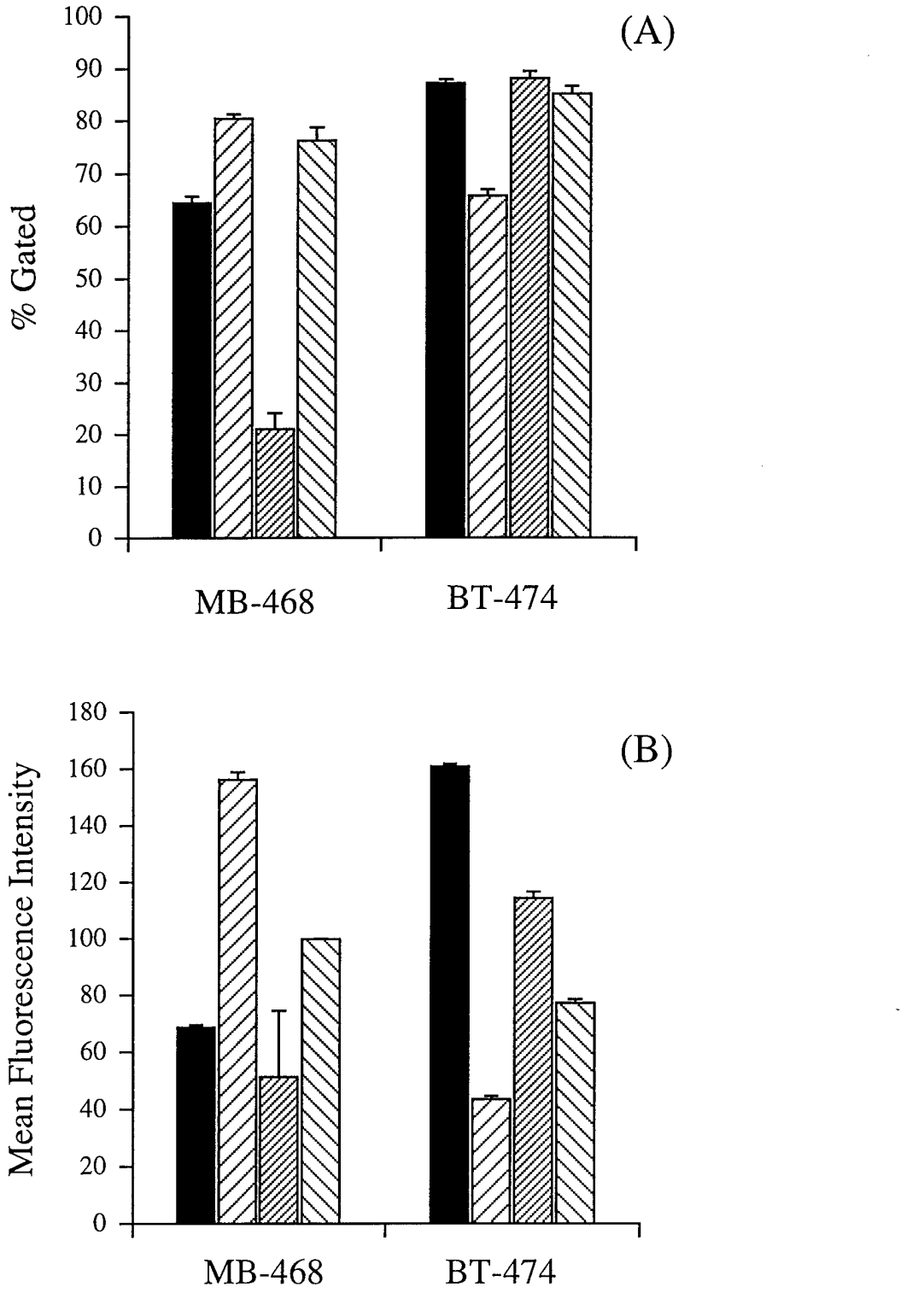
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Figure 5

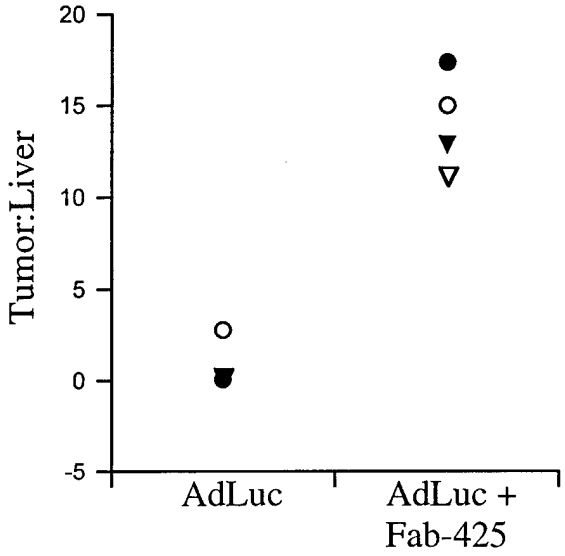


Figure 6

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Applications of vectors in specific diseases or organ systems should be directed to categories C1 - C8.

*A. Viral Vector Development*

- ☐ 1. RNA Virus Vectors
- ☒ 2. DNA Virus Vectors
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*B. Nonviral Vector Development*

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- ☐ 2. Lipid Mediated Gene Transfer
- ☐ 3. Molecular Conjugates

*C. Animal Disease Models and Clinical Applications*

- ☐ 1. Genetic Disease
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- ☐ 3. Cardiovascular
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- ☐ 6. Immunotherapy of Cancer
- ☐ 7. Gene Therapy of Cancer
- ☐ 8. Stem Cell and Blood

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## IMMUNOLOGICAL TARGETING OF ADENOVIRUS TO THE HER-2/neu RECEPTOR OVEREXPRESSED ON BREAST CANCER CELLS


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Adenovirus (Ad) vectors have been widely investigated for cancer gene therapy applications because of their ability to accomplish in vivo gene transfer with high efficiency. However, the broad tropism of Ad also causes the transduction of normal tissues in vivo. We have previously described an immunological method for specifically targeting Ad to tumor associated receptors on cancer cells. This method involves the chemical conjugation of a receptor specific ligand to the Fab fragment of a monoclonal antibody (1D6.14) which neutralizes Ad infection through binding to the Ad fiber knob, preventing binding of Ad to its cellular receptor. The aim of this study was to determine if this strategy can be used to specifically target Ad to the HER-2/neu receptor overexpressed on breast cancer cells. Herceptin is a humanized antibody that binds to the HER-2/neu receptor and is currently being used in clinical trials to treat patients with breast cancer overexpressing HER-2/neu. To determine the level of HER-2/neu expression on BT-474, SK-BR-3, MDA-MB-453, and MDA-MB-231 human breast cancer cells, binding assays with $^{125}$I-labeled Herceptin were performed. This showed that 45%, 47%, 21%, and 4% of the total radioactivity added specifically bound to BT-474, SK-BR-3, MDA-MB-453, and MDA-MB-231 cells, respectively. Infection of these cells with an Ad vector encoding the firefly luciferase gene (AdLuc) showed luciferase expression of 1.6 x $10^5$, 3.7 x $10^6$, 2.3 x $10^4$, and 2.8 x $10^5$ relative light units, respectively. This was inhibited >95% in all cell lines by adding an excess of recombinant Ad5 fiber knob to the cells. Herceptin was conjugated to the anti-fiber knob Fab (Fab-Herceptin) and used to target AdLuc to HER-2/neu receptors on breast cancer cells in vitro. The HER-2/neu positive BT-474, SK-BR-3, and MDA-MB-453 cells had Fab-Herceptin targeted AdLuc to non-targeted AdLuc luciferase expression ratios of 1.0, 1.6, and 0.6, respectively. Targeting AdLuc with Fab-Herceptin to these cell lines was inhibited >70% by incubating the cells with an excess of Herceptin prior to infection. The HER-2/neu negative MDA-MB-231 cells had a targeted AdLuc to non-targeted AdLuc luciferase expression ratio of 0.1, demonstrating the selectivity of the Fab-Herceptin conjugate for targeting HER-2/neu positive cells only. These studies show that Ad can be specifically targeted to the HER-2/neu receptor expressed on breast cancer cells. This strategy should be applicable for selectively treating metastatic breast cancer with Ad vectors carrying therapeutic genes.


TYPE ABSTRACT TO FIT WITHIN THE LINES ABOVE (SEE GUIDELINES FOR ABSTRACT PREPARATIONS)

Era of Hope
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# IMMUNOLOGICAL TARGETING OF ADENOVIRAL VECTORS TO BREAST CANCER CELL LINES

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This research involves the targeting of adenoviral (Ad) vectors to breast cancer cells through immunological methods for gene therapy applications. Ad vectors infect a wide variety of normal tissues, due to the widespread expression of the coxsackie-Ad receptor that binds the Ad knob. The use of the Fab fragment of an anti-knob antibody conjugated to various ligands for targeting adenovirus to cancer cells has previously been described. The goal of this study was to determine if Fab-conjugates can target Ad to the epidermal growth factor receptor (EGFr) or the human epidermal growth factor receptor-2 (HER-2/neu) overexpressed on breast cancer cells.

The murine monoclonal antibody 425 was used to target EGFr and the humanized monoclonal antibody Herceptin (Herc) was used to target HER-2/neu. Each of these ligands were conjugated to the anti-knob Fab fragment to produce Fab-425 and Fab-Herc. The human breast cancer cell lines evaluated in this study were MDA-MB-134, MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, and SK-BR-3. Ads encoding the firefly luciferase gene (AdLuc) or the green fluorescent protein (AdGFP) were used. The optimal AdLuc to Fab-425 or Fab-Herc ratios were determined by mixing various amounts of Fab-425 or Fab-Herc with AdLuc. The mixtures were then used to infect cells in the presence or absence of purified Ad5 knob. The optimal ratio of Fab-425 or Fab-Herc to AdLuc was then used to infect the cell lines in the presence or absence of excess 425 or Herc, respectively, to demonstrate specific infection. A cell mixing experiment was performed with MDA-MB-468 (EGFr+) or BT-474 (HER-2/neu+) in which AdGFP was incubated with Fab-425 or Fab-Herc and added to the cell mixture. GFP expression was determined by FACS 24 h later.

The optimal AdLuc:Fab-425 or Fab-Herc ratio was 1.2 x 106 plaque forming units (pfu) AdLuc: 30 ng Fab-425 or Fab-Herc. The Fab-425 conjugate specifically targeted AdLuc to the MDA-MB-468, MDA-MB-231, and SK-BR-3 cells. Targeting of AdLuc to MDA-MB-468 cells had the best targeting index (Ti; ratio of targeted luciferase expression to non-targeted luciferase expression) of ~5.8. The Fab-Herc conjugate targeted AdLuc to BT-474, SK-BR-3 and MDA-MB-453 cells. The targeting index was ~1.1 for the BT-474 cells and ~1.8 for the SK-BR-3 cells. The cell mixing experiment showed that ~60% of the MDA-MB-468 cells expressed GFP when infected with AdGFP alone. This increased to ~90% when targeted with Fab-425 and decreased to ~30% when targeted with Fab-Herc. The BT-474 cells were ~90% positive for GFP when infected with AdGFP alone, which decreased to ~35% when infected with Fab-425 and returned to ~90% when targeted with Fab-Herc.

In conclusion, the Fab-425 and Fab-Herc were shown to target AdLuc to various breast cancer cells *in vitro*. These conjugates could also target AdGFP to specific cells in a mixed cell population. These studies suggest that the Fab-425 and Fab-Herc could specifically deliver Ad vectors *in vivo* to tumors that overexpress EGFr or HER-2/neu.

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## Bibliography

Meeting abstracts

1.) Abstract presentation at the American Society for Gene Therapy 2nd Annual Meeting, Washington D.C., June 9-13, 1999.

2.) Oral presentation at the Era of Hope Department of Defense Breast Cancer Research Program Meeting, Atlanta, June 8-11, 2000.

Personnel receiving pay from the research effort

Buck E. Rogers